Published in final edited form as:

Anal Chem. 2018 September 18; 90(18): 11016–11024. doi:10.1021/acs.analchem.8b02637.

Chemical Structure and Composition of Major Glycans Covalently Linked to Therapeutic Monoclonal Antibodies by Middle-Down Nuclear Magnetic Resonance

Jiangnan Peng[†], Sharadrao M. Patil[†], David A. Keire^{*,‡}, Kang Chen^{*,†}

[†]Division of Pharmaceutical Analysis, U.S. Food and Drug Administration, Silver Spring, Maryland 20993, United States

[‡]Office of Testing and Research, Office of Pharmaceutical Quality, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, Maryland 20993, United States

Abstract

Glycosylation of monoclonal antibodies (mAbs) is a critical quality attribute that can impact mAb drug efficacy and safety. The mAb glycans are inherently heterogeneous in chemical structure and composition of monosaccharides. The established fluorescence or mass-spectrometry (MS) detection methods for glycosylation evaluation may require multiple steps of glycan cleavage or extensive digestion of the mAb, chemical labeling of the glycans, column separation and report the chemical identity of glycans indirectly through retention time and molecular weight values. In demonstrating chemical structure similarity and comparability among mAb drugs, orthogonal analytical methods for measuring glycan chemistry are needed to ensure the quality of drug products. Here, a “middle-down” NMR method is developed as a proof-of-concept approach to measure the domain-specific glycosylation of marketed mAb drugs without cleavage of the glycan moieties. Complete glycan ¹H/¹³C chemical shift assignments were obtained at ¹³C natural abundance from commercial standard glycans that allowed unambiguous determination of the chemical structure, glycosidic linkage position, and anomeric configuration of each monosaccharide in the major *N*-glycan scaffolds found in mAb molecules. The analysis of glycan anomeric peaks in two-dimensional (2D) ¹H–¹³C NMR spectra yielded metrics for clinically important mAb quality attributes (i.e., galactosylation (Gal%) and fucosylation (Fuc%)), consistent with literature results using a standard glycan-mapping method. Therefore, the middle-down NMR method provided a facile orthogonal measurement for mAb glycosylation

*Corresponding Authors: david.keire@fda.hhs.gov (D. A. Keire), kang.chen@fda.hhs.gov (K. Chen).

Author Contributions

All authors have given approval to the final version of the manuscript.

[†](J.P.) Departments of Chemistry and Biology, Morgan State University, Baltimore, MD 21251.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.8b02637. Information on mAb drug products studied (Table S1); ¹H and ¹³C NMR chemical shifts assignments for glycan standard G2F (Table S2), G0F (Table S3), and G1 (Table S4); demonstrated methods for glycan analysis (Table S5); SEC-FPLC chromatography of digested mAbs (Figure S1); 1D ¹H NMR spectrum of glycan G2F (Figure S2); 2D ¹H–¹³C HSQC and HSQC-TOCSY spectra of standard glycan G2F (Figure S3); 2D ¹H–¹³C HMBC and long-range HSQC spectra of G2F (Figure S4); 2D ¹H–¹³C HSQC spectra of Etanercept Fc and TNF receptor domains (Figure S5) (PDF)

The authors declare no competing financial interest.

characterization with improved chemical information content on glycan structure determination and quantification, compared to standard approaches.

Graphical Abstract

Monoclonal antibody (mAb) drugs are in the mainstream of clinical practice for treatment of a range of diseases, with cancers and autoimmune disorders being the most common targets. As of January 11, 2017, 68 mAb drug products have been approved by the U.S. Food and Drug Administration (FDA),¹ and more are in clinical trials² or being developed as biosimilars. The inherent heterogeneity of mAb glycosylation is one complex aspect of mAb drugs that is important for their safety and efficacy.³ Often, IgG1 mAbs have *N*-glycosylation at the side chain of an asparagine residue in a glycosylation consensus sequence (i.e., Asn-297 of -EEQYNSTYR-) in the Fc domain. The specific chemical structure of the *N*-glycan varies with the eukaryotic cell lines used for recombinant expression of the mAb and the fermentation conditions, e.g., media, pH, temperature, and agitation.^{4–6} Some common glycans in mAb Fc are abbreviated as G0F, G1F, or G2F etc. to indicate differences in monosaccharide composition.⁷ Glycosylation affects protein binding, solubility, stability, pharmacokinetics, and pharmacodynamics (PK/PD), bioactivity and safety (e.g., immunogenicity).^{8–11} Importantly, Fc glycoforms impact antibody structure and effector functions.³ Thus, the glycosylation pattern of a therapeutic mAb is a critical quality attribute (CQA) that is frequently discussed and reviewed.⁷ Overall, glycan structural analysis and heterogeneity control are critical for the quality of all glycosylated therapeutic proteins and are especially important for biosimilar products, where the chemical similarity to the reference product can eliminate or decrease the scope of clinical studies needed for marketing approval.

Currently, high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), fluorescence and combinations of these methods are typically used for glycan characterization, glycosylation site profiling, and intact glycoprotein profiling.^{5,12–15} These methods normally rely on chemical reactions for glycan cleavage or protein digestion, separation, derivatization, and/or reference standards for chemical identity and quantification, e.g., the multiattribute method (MAM)¹⁶ and other high-resolution mass spectrometry methods.¹⁷ The more-advanced MS/MS method can further determine glycan

sequence through permethylation derivatization.¹⁸ While MS is highly sensitive (such that the MS techniques requires less sample than other methods) and is widely used to characterize site-specific post-translational modifications (PTM) in proteins, MS techniques cannot easily measure aspects of glycan chemistry, such as anomeric configuration and linkage positions. Nevertheless, the increased separation power from CE and hydrophilic interaction liquid chromatography (HILIC) HPLC columns overcomes most of the isobaric glycan identification limitations of MS. In addition, the combination of LC, MS and/or fluorescence labeling greatly improves the method sensitivity in glycan detection and the precision of their quantification. The LC- and MS-based methods have been assessed sufficiently robust to be used as batch release methods when protocols were established and followed.^{19,20} However, because of the importance of glycan measurements to mAb quality, multiple orthogonal analytical methods capable of identifying the position and anomeric configuration of linkages between monosaccharides or the stereochemistry of monosaccharides in polysaccharide chains are suggested, to ensure the glycan structure characterization for either similarity or comparability evaluation in regulatory applications.²¹ The NMR method proposed in this work represents a new approach for glycan analysis that compliments other available techniques.

NMR has been extensively applied in characterizing the chemical structure and dynamics of polysaccharides²² and glycosylated mAbs.²³ Two-dimensional (2D) NMR techniques have been used to determine the composition and sequence of the *N*-linked glycans in small proteins²⁴ and digested peptides.²⁵ NMR, in combination with MS²⁶ and LC,²⁷ was used to characterize free glycans. The NMR/LC method was applied to characterize the free *N*-glycans released from the cetuximab.²⁸ Recently, 2D ¹H–¹³C NMR experiments were shown to identify and assign chemical shift of glycans in proteins without chemical cleavage of the glycans from the glycoprotein.²⁹ However, high-resolution 2D NMR with the protein denaturation method has not been demonstrated on any protein therapeutics, especially mAb drugs. In addition, recent MS studies have shown that the hinge-region-specific enzymes (IdeS) can reproducibly cleave mAbs to defined domains and has been called middle-down MS.^{30,31} Here, 2D ¹H–¹³C heteronuclear single quantum coherence (HSQC) NMR spectra were collected on similar IdeS-cleaved mAb fragments as those obtained for middle-down MS with the added presence of chemical denaturants. The resulting glycan anomeric nuclei resonance data from the “middle-down NMR” measurements provided the exact chemical structures and populations of major mAb glycans, creating a new method for mAb drug quality assessment.

EXPERIMENTAL SECTION

Materials.

Seven mAb drug products—including three brands of rituximab, one infliximab, one bevacizumab, one etanercept, and one adalimumab—were sourced from the U.S. and Indian markets (see Table S1 in the Supporting Information). Standard glycans G2F (NA2F, 110 μ g), G0F (NGA2F, 70 μ g), G1 (NA2G1, 70 μ g), MAN5 (60 μ g), and MAN6 (60 μ g) were purchased from Prozyme (Hayward, CA).

NMR Sample Preparations.

The standard glycan was dissolved directly in 250 μL of D_2O and transferred to a Shigemi NMR tube. Drug products of Remicade, Humira, and Enbrel were prepared at 10 mg/mL by adding pure water (Quality Biological). Other mAb drug products were used directly as formulated at a concentration of 10 mg/mL. Each drug solution (6 mL) was incubated with 30 μL (1500 U) of IdeS FabRICATOR (Genovis, Cambridge, MA) overnight at room temperature. The digested mAb samples were then separated (Figure S1 in the Supporting Information) on a Superdex 75 pg 16/600 column through an FPLC separation system (GE Healthcare). Fractions corresponding to domains Fab and Fc were separately pooled and concentrated to 0.5 mL using a 3-kDa cutoff ultracentrifuge tube (Millipore Corporation), then washed with 4.5 mL of pure water. The Fab and Fc samples were lyophilized and dissolved in 0.45 mL of 7.8 M urea- d_4 (Cambridge Isotope Laboratories, Inc.) prepared in D_2O . Another 0.05 mL of 20 mM dithiothreitol- d_{10} was added to form the final NMR sample.

NMR Data Collection.

NMR spectra were obtained on a Bruker Ascend 850 MHz spectrometer equipped with a TCI cryoprobe. All measurements were carried out at 25 $^{\circ}\text{C}$. The HSQC pulse program is a slight modification of Bruker standard pulse sequence *hsgcetgpsi2* and the $^1J_{\text{HC}}$ coupling constant was set at 170 Hz. The complex data points of 1024 were collected for both the ^1H and the ^{13}C dimensions. The ^{13}C spectral width was 100 ppm, and the carrier was at 95 ppm. The ^1H spectral width was 14 ppm, and the carrier was at 4.7 ppm. The recycle delay was 1.5 s with 24 scans averaged for each free induction decay (FID), and the total acquisition time was 11 h. The data were processed using MestReNova 11.0.3 or NMRPipe software. Each FID was apodized with a cosine function, and the first data point was multiplied by 0.5. Zero filling was up to 4096 points in the ^1H dimension and 2048 points in the ^{13}C dimension. The spectra baseline correction was performed using polynomial fit. The ^1H chemical shift was referenced to internal TSP, and ^{13}C chemical shift was referenced with calculation.³²

RESULTS

To our knowledge, a complete and unambiguous chemical shift assignment of all ^1H and ^{13}C nuclei of the standard glycans G0F, G2F, and G1 is not available in the literature. Chemical shift assignments on similar octasaccharide and decasaccharide glycans have been reported.³³ Here, complete NMR ^1H and ^{13}C chemical shift assignments for the most common standard glycans found on IgG1 were determined on commercially available glycan standards. Then, chemical shift assignments for mAb major and minor (high-mannose) glycans were established by comparing them to standard glycans. In this way, the 2D NMR anomeric fingerprint region signals of marketed mAb glycans were assigned. The anomeric region is informative because its pattern can be used to identify the chemical structure and composition of the major and minor glycans, as well as to quantify the galactose-6 (Gal6) and fucose (Fuc) levels for different mAb drugs, which are critical quality elements for mAb drugs.

Chemical Shift Assignments for Glycan Standards G0F, G2F, and G1.

Glycans are composed of monosaccharides linked through glycosidic bonds (Figure 1). Each monosaccharide in a chain is an isolated NMR spin system bounded by ether linkages. Unambiguous assignments of all ^1H and ^{13}C nuclei of monosaccharides of G2F (Table S2 in the Supporting Information), G0F (Table S3 in the Supporting Information), and G1 (Table S4 in the Supporting Information) were achieved by the analysis of heteronuclear single quantum coherence (HSQC), heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY), TOCSY, heteronuclear multiple bond correlation (HMBC), and correlation spectroscopy (COSY) spectra of glycan standards. Although the ^1H chemical shifts of each spin system can be assigned by 2D ^1H - ^1H TOCSY, significant overlap of nonanomeric ring protons (from 3.5 ppm to 4.0 ppm; see Figure S2 in the Supporting Information) prevented unambiguous assignment. Therefore, the overlap was resolved using 2D ^1H - ^{13}C HSQC-TOCSY spectra (Figure S3 in the Supporting Information), where overlapped TOCSY correlations were resolved via the ^{13}C chemical shifts. The ^1H and ^{13}C chemical shifts are sensitive to the linkages and their configuration. The complete chemical shift assignments allowed the identification of the anomeric configuration and the glycosidic linkage position for each monosaccharide.

α and β Configuration of Monosaccharides.

In all glycan standards, the anomeric OH of *N*-acetylglucosamine-1 (GN1) is not covalently linked to an asparagine side-chain amide group, as GN1 would be in a mAb; therefore, in the standards, two sets of resonances corresponding to the anomeric isomerization of GN1 (α and β) were observed (see Figures S2 and S3, and Tables S2–S4 in the Supporting Information). For G2F, the ^1H signal at δ 5.189 ppm was assigned as the α anomer, because of the small coupling constant $^3J_{\text{H1},\text{H2}}$ value of 3.3 Hz, while the ^1H signal at δ 4.703 ppm was assigned as the β anomer, because of the large coupling constant ($^3J_{\text{H1},\text{H2}} = 8.3$ Hz). Based on the HSQC peak intensities of the GN1 anomeric resonances, the population ratio of α/β isomer was 1.78:1. Similar anomeric isomerization ratios were observed for the G0F and G1 standards.

The α/β isomer equilibrium of GN1 resulted in two sets of resonances for the fucose (Fuc) monosaccharide, e.g., HSQC peaks of 102.1/4.903 and 102.3/4.897 ppm were observed for the anomeric C1/H1 pair of the fucose. The two peaks had intensity ratio of 1.65:1, similar to that of the GN1 α/β (1.78:1). Thus, the stronger peak at 102.3/4.897 ppm was assigned to the fucose linked to the α isomer of GN1, and the peak at 102.1/4.903 ppm was assigned to the fucose linked to the β isomer of GN1. Only one set of resonances in HSQC and HSQC-TOCSY spectra was observed for *N*-acetylglucosamine-2 (GN2), indicating that the GN1 isomerization impact on the chemical shifts of GN2 was too small to be distinguished. The chemical shifts of the remaining monosaccharides were not affected by GN1 isomerization.

The assignment of anomeric configuration of monosaccharides using J -coupling constants³⁴ and ^1H and ^{13}C chemical shifts is well-established.^{35–38} Overall, the equatorial H-1 (α anomer) of hexopyranosides generally resonates downfield at δ 4.8–5.3 ppm, while the axial (β anomer) H-1 resonates at a field 0.3–0.5 ppm higher (4.4–4.8 ppm).³⁵ Thus, the anomeric configuration of each monosaccharide in the glycan standards can be readily determined by

the ^1H chemical shifts using the above criteria. The β anomeric configurations were observed for all monosaccharides, except for the α anomeric configuration in Fuc, Man4, and Man4' (see Figure 1a).

Glycosidic Linkage Positions of Monosaccharides.

The covalent ether bond formed between one saccharide and its neighboring saccharide causes a 4–10 ppm downfield shift of the anomeric ^{13}C chemical shift, compared to a free monosaccharide. Thus, the linkage position can be determined by the comparison of the chemical shifts of each monosaccharide to those of free monosaccharide reported in the literature.³⁹ Here, the linkage carbons were mapped to C4 and C6 of GN1, C4 of GN2, C3 and C6 of Man3, C2 of Man4 and Man4', and C4 of GN5 and GN5' of standard glycans. To establish the sequence of each monosaccharide, an HMBC experiment was performed (see Figure S4 in the Supporting Information). The HMBC correlations of GN2-H1-GN1 β -C4, Man3-H1-GN2-C4, Man4-H1-Man3-C3, Man4'-H1-Man3-C6, GN5/5'-H1-Man4/4'-C2, and Gal6/6'-H1-GN5/5'-C4 established the β -1,4 linkage of GN2 to GN1 β , β -1,4 linkage of Man3 to GN2, α -1,3 linkage of Man4 to Man3, α -1,6 linkage of Man4' to Man3, β -1,2 linkages of GN5/5' to Man4/4' and the β -1,4 linkages of Gal6/6' to GN5/5', respectively. The correlations of GN2-H1 to GN1 α -C4 and Fuc α / β -H1 to GN1 α / β -C6 were either extremely weak or not observed, likely due to chemical exchange between isomers. The long-range H-C correlation was also explored using HSQC experiment by setting the coupling constant to 6 Hz (see Figure S4) and similar correlation peaks were observed.

Structure and Compositions of Major Glycans on the mAb-Fc Domains.

Based on the NMR analysis of glycan standards, the chemical shift values of glycans range from 3.4–5.3 ppm and 54–107 ppm for ^1H and ^{13}C , respectively, except for the H6/C6 of fucose and acetyl of *N*-acetylglucosamine. Because of the absence of a large structural change in the glycans for free versus protein-linked glycans, most of the 2D $^1\text{H}/^{13}\text{C}$ assignments of the free glycan standards were transferable to glycans covalently linked to mAb. A total of seven mAb drugs were analyzed, including rituximab from three manufacturers, infliximab, bevacizumab, etanercept, and adalimumab (see Table S1 in the Supporting Information). The three brands of rituximab allowed the assessment of glycosylation similarity; the five different mAbs allowed the evaluation of method applicability to a wider range of mAbs. To measure domain-specific glycosylation results, a full-length mAb was digested to Fc and (Fab')₂,³¹ then NMR data were collected on these domains after separation. The HSQC spectrum of the denatured rituximab-Fc domain is shown in Figure 2. The denaturation will change the chemical shifts of secondary structure sensitive nuclei $^{13}\text{C}\alpha$ (50–70 ppm) and ^{13}CO (170–180 ppm) of protein backbone, but does not significantly affect amino acid side chains or glycan resonances. Protein signals can overlap with some C2 and C6 resonances of monosaccharides in the range of 55–65 ppm (^{13}C), but the protein signals are less common above 70 ppm. Indeed, most glycan signals in the blue box (^{13}C of 55–110 ppm) of Figure 2 were separated from protein signals, because of the difference in ^1H and/or ^{13}C chemical shifts. The anomeric signals (^{13}C of 100–110 ppm), except for GN1, are illustrated in the red box in Figure 2. By comparison with the reference glycan standards G0F, G1, and G2F spectra, the chemical shifts of most of the ^1H and ^{13}C nuclei of each monosaccharide of rituximab from Rituxan could be assigned (see

Table 1). All monosaccharide units—GN1, GN2, Man3, Man4, Man4', GN5/5', Gal6/6' and Fuc—were identified. However, the unambiguous assignment of H6/C6 of GN2, GN5/5' a, GN5/5' b, and Gal6/6' was not achieved, because of overlap with protein signals.

The anomeric configurations of GN2, Man3, Man4, Man4', GN5/5', Gal6/6', and Fuc were determined by the assigned anomeric ^1H and ^{13}C chemical shifts and found to be consistent with the glycan standard. The monosaccharide GN1 was covalently attached to the side-chain amide group of Asn297; thus, its ^1H chemical shift value of 5.083 ppm indicated a β anomeric configuration by comparison to the ^1H chemical shift value of 5.07 ppm reported for the GN1 β anomeric configuration in a model asialo-glycan-Asn compound, where the β anomeric configuration was assigned using the coupling constants.⁴⁰

The linkage position of each monosaccharide can also be determined by the ^{13}C chemical shifts of the linkage carbons, i.e., GN1-C4,6, GN2-C4, Man3-C3,6, Man4-C2, Man4'-C2, and GN5-C4. The chemical shifts of each mAb monosaccharide were comparable with those for the glycan standards of G2F and G0F, suggesting that the same linkages were present in the glycans of the Fc domain and the in the G0F, G1F, and G2F standards. In a similar fashion, the saccharides of the Fc domain of six other mAbs were identified. All mAbs analyzed contained GN1, GN2, Man3, Man4, Man4', GN5/5', Gal6/6', and Fuc as their major scaffold monosaccharide components. This straightforward readout by chemical shift compares favorably with the much more difficult process of assigning glycan linkages and configurations by MS methods.

Identification of Minor Glycans.

The fingerprint anomeric crosspeaks were in a narrow window between 4.4–5.3 ppm in the ^1H dimension and 98–107 ppm for ^{13}C dimension of the HSQC spectrum, where all signals had a spectral S/N of 10 or greater (see the red box in Figure 2). Because the signals in this region are specific for the anomeric nuclei of linked monosaccharides and there is no overlap with any other signals, the anomeric crosspeaks in ^1H – ^{13}C -HSQC were used to compare the glycan species among different mAb drug products.

The spectra for the anomeric region of each mAb are shown in Figure 3. The anomeric signals of the three rituximab products were very similar, except for a minor crosspeak *a* at 4.917 ppm for ^1H and 102.12 ppm for ^{13}C . Ristova showed the lowest relative intensity of peak *a*, while the Reditux showed the strongest intensity of peak *a* when both spectra were plotted in the same S/N threshold. Despite the intensity differences, all three rituximab products were visually similar in glycosylation pattern, indicating the similar glycan composition and structure. The signals for major monosaccharides, GN1, GN2, Man3, Fuc, Man4, Man4', GN5/5' a, GN5/5' b, and Gal6/6', were similar for all seven mAb products. In addition to the minor peak *a*, five more minor peaks, labeled *b*–*f*, were observed for the Fc domains of the other four mAbs products, i.e., Avastin, Remicade, Humira, and Enbrel (see Figure 3). Three additional minor peaks—*b*, *c*, and *d* at 4.914/102.39, 4.877/102.56, and 5.108/105.20 ppm, respectively—were observed for infliximab Fc (Remicade). For etanercept-Fc (Enbrel), peaks *b*, *c*, *d*, and an additional minor peak (*e*) at 5.13/105.39 ppm were found. For adalimumab-Fc (Humira), all the above minor peaks and an extra peak (*f*) at 5.070/105.01 ppm were observed. The minor signals *a*, *c*, and *d* corresponded to the

anomeric groups of ManB, Man4', and ManA of the high mannose glycans MAN-5 and MAN-6, respectively (see Figure 1b), while the minor signals *e* and *f* corresponded to the anomeric of Man4 of the high mannose MAN-5 and MAN-6, respectively. Of note, the minor signal *b* was not assigned, because of the lack of a corresponding peak in the standard glycans used in this work.

Quantifying Galactosylation and Fucosylation Levels.

The terminal galactose content of mAb glycans has been reported to impact the complement-dependent cytotoxicity (CDC) of mAbs.^{2,41} Notably, a previous investigation of six marketed mAb products indicated large variations in galactosylation levels.⁴² In addition, a lack of fucosylation has been observed to markedly increase antibody-mediated cellular cytotoxicity (ADCC) via increased affinity for FcγRIIIa.^{43–45} The enhanced ADCC associated with low levels of fucosylation may result in toxicity that is due to off-target binding and can be a potential safety concern for a therapeutic mAb.⁴⁶ Therefore, the galactose and fucose content of therapeutic mAbs must be accurately assessed to ensure the safety of these drugs.

To measure the galactosylation level using NMR, the anomeric peak ratio of Gal6/Man4, normalized by the corresponding ratio in standard G2F, was used. The NMR peak ratio method reduced the differences in dynamics of individual monosaccharides and eliminated effects from concentration differences from sample to sample. The levels of galactosylation for all seven mAb products were calculated (see Table 2). Three brands of rituximab products—Rituxan, Reditux, and Ristova—showed the highest level of galactosylation, 44%–47%, indicating high percentages of G1F and G2F in their glycan pools. Etanercept showed the next-highest galactosylation of 42% ± 1%. Infliximab showed less galactosylation (38% ± 1%) than rituximab, which was consistent with previous measurements using an orthogonal method (i.e., top-down intact-MS).³¹ Bevacizumab and adalimumab showed the least galactosylation, with 7.3% ± 0.7% and 15% ± 1%, respectively, indicating that G0F was the predominant glycan for these two mAbs. For the U.S.-marked mAb drug products Rituxan, Remicade, Avastin, and Humira, the agreement on the galactosylation level were within 2% between the values measured by the current middle-down NMR and the free glycan methods published by other scientists (see Table 2).^{47–50} One exception was Enbrel, where the Fc galactosylation level from the free-glycan method was not available; instead, the results were recalculated from the glycopeptide method⁵¹ and differed by 13% from the middle-down NMR measurement of 42%. The larger difference might originate from error associated with recalculation of the Gal% data from the raw plots.⁵¹

The level of fucosylation of seven mAb products were calculated by direct peak intensity ratios between Fuc and Man4 (see Table 2). A level of fucosylation of 77%–87% was observed for all seven tested mAbs, which is consistent with most mAb glycans being fucosylated.⁵² Three brands of rituximab products—Rituxan, Reditux, and Ristova—showed a similar level of fucosylation (83%–87%). Infliximab, bevacizumab, and etanercept showed a lower level of fucosylation (77%–80%). The middle-down NMR results on fucosylation are consistently 10%–18% lower than the results from the free-glycan^{47–50} or

glycopeptide⁵¹ methods. The 10%–18% differences may be attributable to differences in dynamics between glycan monosaccharide Fuc and Man4 units located closer and further away, respectively, from the protein linkage. Thus, the Man4 unit further up the glycan chain has more flexibility, compared to the more rigid—and, therefore, lower intensity—signal of Fuc closer to the asparagine side chain, which decreases the NMR measured Fuc%. The intensity differences caused by dynamics could be mitigated if the Fuc% value can be calibrated using a standard glycoprotein with 100% fucosylation as a comparator.

Non-Fc Domain Glycans.

In addition to the conserved Fc glycans, 15%–25% of human serum IgG contains glycans covalently linked to the variable domains.⁵² 2D HSQC spectra for the non-Fc (Fab) domains of all seven drugs were also measured. Among them, only the HSQC spectra of the TNF receptor of etanercept had crosspeaks in the anomeric region (see Figure 3). Strong crosspeaks at δ 101.44/4.958 (*g*), 105.64/4.532 (*h*), and 107.48/4.524 (*i*) were observed. These signals were not observed for the Fc domains of the other mAb drugs and may arise from *O*-glycans in etanercept. Among all the tested mAb products, only the Fc-fusion protein etanercept was reported to carry *O*- and *N*-glycan sites in the linker and TNF- α regions.⁵³ The sialic signals, δ 42.6/2.8 and 42.6/1.8 ppm (Figure S5 in the Supporting Information) corresponding to C3–H3 resonances of sialic acid,⁵⁴ were observed only in the spectrum of TNF receptor of etanercept. The chemical shift separation between the axial and equatorial proton was 1 ppm, corresponding to α anomeric configuration in C2.⁵⁴ The NMR glycosylation profile of non-Fc regions should be another important quality attribute for mAb products, and the facile assignment of these nonstandard glycan configurations is an improvement over existing methods.

CONCLUSIONS AND DISCUSSIONS

Analytical Methods for mAb Glycan Characterization.

Table S5 in the Supporting Information lists selected published analytical methods for mAb glycosylation identification and quantification. The intact MS method, involving the least sample preparation steps, allows the identification of glycan pairing patterns. However, the intact method only measures major glycan forms (e.g., fucosylated glycans).^{55,56} The middle-down method has better differentiability for nonfucosylated glycans (i.e., G1 and G2 can be identified and quantified).⁵⁵ In one comparison study, the middle-down MS method, not the bottom-up glycopeptide method, was demonstrated to achieve glycan quantification results that were consistent with the free-glycan method values.⁵⁵ However, in other comparison studies,¹⁵ including the more recent development of the MS based multiattribute method (MAM),^{16,57} the glycopeptide methods was shown to yield consistent values to the free-glycan method. Notably, for the standard free-glycan methods, most development work has centered on LC columns that can improve the separation of glycans.¹⁹

The Current “Middle-Down” 2D NMR.

Direct NMR measurement of ¹³C-labeled mAb glycan signals have been made for the Fc domain²³ or glycopeptide bearing glycans.⁵⁸ What is unique here is the use of 2D ¹H–¹³C NMR to study glycans attached to a denatured mAb at ¹³C natural isotopic abundance of

1.1%, which makes this method amenable to the characterization of mAb therapeutics. Generally, the NMR spectra with sufficient S/N for analytical assessment require high solute concentration and fast molecular dynamics on a nanosecond (ns) time scale or faster. However, the mAb-Fc domain in its native folding state is not an ideal sample for NMR data collection, because the Fc domain is actually a (Fc)₂ dimer ~50 kDa in size³¹ (see Figure S1 in the Supporting Information) with limited solubility (<20 mg/mL for Fc). In addition, the glycan moieties undergo microsecond to millisecond exchange broadening²³ and are buried in an interdomain cavity hindering observations of these signals. Here, the mAb middle-down method³¹ with denaturation²⁹ increased the glycoprotein solubility (>40 mg/mL for Fc) and liberated the glycans, such that they exhibited fast nanosecond dynamics. Furthermore, the conservation of the protein-glycan linkage provides the advantages of less experimental variation from sample preparation steps, expands applicability to PNGase-resistant glycoproteins,^{59,60} and, overall, offers more chemical quality attributes for evaluation.

The NMR-measured glycan ¹H and ¹³C chemical shift values unambiguously determined the chemical structure of major monosaccharide components of the glycans, including their linkage position and configuration on seven marketed mAb drug products with reasonable sample requirements. In addition, the middle-down NMR method measured domain-specific glycosylation patterns. In this work, only etanercept has NMR measurable “Fab” glycans, including sialic acid. The inspection of chemical shift values obtained on Fc domains showed that the glycan scaffold structures are conserved, mainly G2(F)/G1(F)/G0(F), among different mAb drugs. Furthermore, the anomeric crosspeaks for minor glycans showed patterns that identified high mannose glycans Man5 and Man6. Importantly, the NMR intensities were low for these species, because the mAbs observed with high mannose modifications have been shown to increase clearance and lead to PK differences.⁶¹

In addition to glycan identification, the relative quantities of galactose and fucose were calculated for the glycan mixture of each mAb drug product. The results indicated that glycans of all seven mAb drug products were galactosylated in the range of 7%–47% and fucosylated in the range of 77%–87%. The results were consistent with the results from the free-glycan and glycopeptide methods (recall Table 2). To obtain values for Gal% and Fuc% or other minor glycans down to a minimal abundance of 10%, as an estimated limit of quantification (LOQ), ~30 mg of mAb with 11 h of NMR time on a cryogenic probe are needed. The ¹H–¹³C NMR sample amount is much higher than the sample requirements of 0.1 mg and 0.2 mg for the free-glycan⁴⁸ and glycopeptide⁵¹ methods, respectively.

The Future of mAb Glycan Quality Research and Control from NMR Data.

Recent NMR studies have shown that NMR spectra collected on a protein therapeutic across instruments, field strengths, and laboratories are highly reproducible and precise.^{62,63} Here, most of the NMR chemical shift assignments that were obtained from standard glycans⁶⁴ do not change when the glycans are covalently linked to proteins, except for those nearest to the linkage. Importantly, the shifts are fundamental atomic properties that do not vary across measurements. Therefore, the possibility exists that a library spectrum acquired once in one location can be compared years later with a new sample of the same material acquired on a

different instrument in a different location for comparability purposes without the requirement of a standard for comparison. Such instrumental performance is ideal for quality assurance purposes over the lifecycle of complex drugs such as protein therapeutics.

The NMR protocol described here provides chemical structure information for complex mAb glycans. Clearly, the anomeric 2D NMR fingerprint signals could be used as markers to identify the chemical similarities or differences in the glycosylation of drug products between batches and brands. Because the measurement is independent of the mAb-glycan linkage type (e.g., *N*-linked or *O*-linked), the NMR glycan profiling can identify unknown or unexpected monosaccharides that may be critical for drug development, quality control or surveillance. Such measurements are key because glycan heterogeneity is directly associated with mAb drug safety and efficacy.⁵

In conclusion, application of modern high-resolution 2D NMR experiments have been demonstrated for the resonance assignment and elucidation of the chemical structure of major monosaccharides in marketed mAb drugs. The proposed NMR method is universally applicable, highly precise, informative, and transferable, and, as such, should be considered as an additional tool to ensure the quality of glycosylated protein therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This article reflects the views of the author and should not be construed to represent the U.S. FDA's views or policies. We thank Daro Freedberg and Marcos Battistel for their helpful discussions and Maria Gutierrez Lugo for critical reading of the manuscript. Support for this work from the U.S. FDA CDER Critical Path funds is gratefully acknowledged. This project was supported, in part, by an appointment (J.P. and S.M.P.) to the Research Participation Program at the CDER administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy and the U.S. FDA.

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Figure 1. Structure illustrations for standard glycans. (a) Molecular structure of G2F. For GN1, the β configuration was drawn, but both α and β configurations are in equilibrium for free glycan. Man4', GN5', and Gal6' represent the α 1– α 6 branched saccharides from Man3. (b) Symbolic representation of common glycans found in mAbs.

Figure 2. Full (left) and the expanded glycan region (right) of 2D ^1H - ^{13}C HSQC spectrum of rituximab from Rituxan. Blue box indicates glycan signal range; red box indicates fingerprint anomeric signals from glycans.

Figure 3.

Fingerprint anomeric regions of HSQC spectra of all studied mAb drugs. All spectra, except the bottom right one, were obtained from the Fc domains of the mAbs. The plotting threshold of the signal-to-noise (S/N) ratio was 10 for all spectra. GN5/5'a indicated galactosylated GN5/5', while GN5/5'b indicated the terminal GN5/5'. The crosspeaks *a*, *c*, and *d* were respectively assigned to the anomeric peaks of ManB, Man4', and ManA of the high mannose glycans MAN-5 and MAN-6. The crosspeaks *e* and *f* were anomeric peaks of Man4 of the high mannose MAN-5 and MAN-6, respectively. The crosspeaks *b*, *g*, *h*, and *i* were not assigned.

Table 1.Chemical Shift Assignment of the Glycans from Fc Domain of Rituximab^a

nucleus	GN1	GN2	Man3	Fuc	Man4	Man4'	GN5/5', ^b _a	GN5/5', ^b _b	Gal6/6'
C1	81.16	104.0	103.5	102.1	102.4	99.72	102.5	102.7	106.0
C2	57.59	57.66	72.98	71.04	79.36	79.49	57.61	58.10	73.75
C3	75.58	74.79	83.56	72.26	72.33	72.39	74.67	76.17	75.42
C4	81.28	82.59	68.29	74.98	70.16	70.17	81.73	72.75	71.42
C5	77.82	77.16	77.16	69.68	76.50	75.72	77.52	78.72	78.22
C6	69.49	62.75	68.44	<i>c</i>	64.52	64.32	62.77	<i>c</i>	63.42
H1	5.083	4.656	4.759	4.834	5.158	4.919	4.553	4.537	4.466
H2	3.802	3.759	4.277	3.807	4.214	4.098	3.737	3.738	3.588
H3	3.756	3.738	3.791	3.924	3.907	3.860	3.705	3.586	3.684
H4	3.840	3.729	3.857	3.792	3.519	3.518	3.719	3.471	3.940
H5	3.602	3.596	3.650	4.155	3.789	3.609	3.593	3.457	3.734
H6a	3.914	3.853	3.978	<i>c</i>	3.959	3.910	3.979	<i>c</i>	3.931
H6b	<i>c</i>	<i>c</i>	3.786	<i>c</i>	3.629	3.656	<i>c</i>	<i>c</i>	3.773

^aChemical shift assignments have been derived from the data of the Fc domain of Rituxan.^bGN5/5',^a is GN5 with galactosylation; GN5/5',^b is GN5 without galactosylation.^cGlycan resonances werenot assigned due to low intensity or overlap with protein signals.

Table 2.
Comparison between NMR Measured Levels of Galactosylation and Fucosylation and Other Methods

mAb	drug product	Galactosylation (%)			Fucosylation (%)		
		Gal6/Man4	by middle-down NMR ^a	by other methods ^c	Fuc/Man4	by middle-down NMR	by other methods ^c
Rituximab	Rituxan	0.981 ± 0.016	46 ± 1	46.17–56.08 (ref 47)	0.829 ± 0.101	83 ± 10	96.97–97.78 (ref 47)
Rituximab	Ristova	1.00 ± 0.026	47 ± 1		0.857 ± 0.049	86 ± 5	
Rituximab	Reditux	0.937 ± 0.020	44 ± 1		0.871 ± 0.087	87 ± 9	
Infliximab	Remicade	0.801 ± 0.029	38 ± 1	39.1 ± 3.6 (ref 48)	0.796 ± 0.097	80 ± 10	90.0 ± 1.7 (ref 48)
Bevacizumab	Avastin	0.156 ± 0.014	7.3 ± 0.7	8.7–21.8 (ref 49)	0.783 ± 0.071	78 ± 7	96.2–98.3 (ref 49)
Etanercept	Enbrel	0.900 ± 0.023	42 ± 1	29 (ref 51)	0.770 ± 0.058	77 ± 6	87 (ref 51)
Adalimumab	Humira	0.315 ± 0.023	15 ± 1 ^b	17.6–21.6 (ref 50)	0.819 ± 0.169	82 ± 17 ^b	98.3–98.9 (ref 50)

^aThe percentage of galactosylation was normalized against the Gal6/Man4 peak ratio (2.133) of G2F, which is 100% galactosylated.

^bVariation measured from repeating the experiments 1 year apart.

^cAll data, except for Etanercept, were taken from references and measured using the free-glycan (glycan-mapping) method. The Etanercept data were recalculated from a bar plot in ref 51, based on the glycopeptide method.